Methylation profiles of genes utilizing newly developed CpG island methylation microarray on colorectal cancer patients

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ABSTRACT

Aberrant methylation of DNA has been shown to play an important role in a variety of human cancers, developmental disorders and aging. Hence, aberrant methylation patterns in genes can be a molecular marker for such conditions. Therefore, a reliable but uncomplicated method to detect DNA methylation is preferred, not merely for research purposes but for daily clinical practice. To achieve these aims, we have established a precise system to identify DNA methylation patterns based on an oligonucleotide microarray technology. Our microarray method has an advantage over conventional methods and is unique because it allows the precise measurement of the methylation patterns within a target region. Our simple signal detection system depends on using an avidin–biotinylated peroxidase complex and does not require an expensive laser scanner or hazardous radioisotope. In this study, we applied our technique to detect promoter methylation status of O[6]-methylguanine-DNA methyltransferase (*MGMT*) gene. Our easy-handling technology provided reproducible and precise measurement of methylated CpGs in *MGMT* promoter and, thus, our method may bring about a potential evolution in the handling of a variety of high-throughput DNA methylation analyses for clinical purposes.

INTRODUCTION

It is now clear that aberrant methylation of CpG island-stretches within a promoter, causing silencing of tumor suppressor genes, is a widespread phenomenon in cancer cells (1). Abnormal methylation also occurs at CpG-rich regulatory elements in intronic and coding parts of genes in certain tumors (2). At the same time, genome-wide hypomethylation of DNA is generally observed in tumor cells. Therefore, cancer can be understood not only as a genetic but also as an epigenetic disease.

Methylation density in promoter CpG islands was indicated to be important for gene silencing rather than methylation that occurred at a limited number of CpG islands in the promoter (3). Since tumors can progressively but differently accumulate methylation at the promoter CpG islands of multiple cancer-related genes, understanding and measurement of promoter methylation patterns on each gene is also required. The technique commonly used for the methylation analysis is based on the bisulfite modification of the genomic DNA. Since sodium bisulfite treatment exclusively converts unmethylated cytosine to uracil under appropriate conditions (4), subsequent analysis to differentiate unconverted cytosine from converted uracil enables us to know the primary methylation status. Methylation-specific PCR (MSP) (5) is widely used to analyze promoter methylation, but only a limited number of CpG islands can be analyzed by this method. Accordingly, it does not fulfill the need to quantify the level of methylation relating to the gene silencing. Conversely, bisulfite DNA sequencing (6–8) provides precise methylation status over an amplified region, but it requires large-scale sequencing of multiple plasmid clones. Several groups have recently shown that the methylation status can be precisely achieved by microarray-based technologies (9–12). These assays can be applied to analyze number of CpG island methylation of multiple genes at the same time. There, however, remains a requirement to develop an inexpensive and high-throughput but still quantitative method to screen the methylation status of multiple genes, especially for the purpose of medical diagnosis.

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We have recently modified the original microarray strategy and developed an alternative microarray technique that allows immobilization of non-modified oligonucleotides on solid support by using the polycarbodiimide-coated slide (13). This results in a substantial cost saving in the fabrication of an array. Therefore, we applied this method for the CpG island methylation microarray, which can efficiently scan the methylated cytosines in tumor genome.

In this study, we describe a novel microarray procedure and its application for the mapping of methylated CpG islands in human O^6-methylguanine-DNA methyltransferase (*MGMT*) gene in the properties of colorectal cancers (CRCs) (3). The technology uses an avidin–biotinylated peroxidase complex (ABC) method to visualize hybridization signals (14,15) and the high-quality methylation microarray signals can be detected with good reproducibility. This detection system does not rely on an expensive fluorescence detection system or hazardous radioisotope. The detection of methylation profiles by the ABC method, without compromising the quality of the DNA chip, results in a further substantial cost saving. Furthermore, our hybridization and detection procedures can be achieved within 2 h, whereas widely used microarray technologies are time-consuming and takes up to 10 h. The approach described here provides an effective tool, which produces specific and reproducible hybridization signals in DNA-chip methylation profiling.

MATERIALS AND METHODS

Synthesis of oligonucleotide

Oligonucleotides labeled with 5′-biotin were synthesized on an ABI 3900 DNA synthesizer at a 0.2 μmol scale using the standard phosphoamidite method. The labeled oligonucleotides were purified by reverse-phase high-performance liquid chromatography using a standard procedure and dried *in vacuo*. Non-modified oligonucleotides were also synthesized on an ABI 3900 DNA synthesizer at a 0.2 μmol scale using the standard procedures. The non-modified oligonucleotides were then purified on a reverse-phase cartridge following a standard procedure and dried *in vacuo*.

Human tissue samples and DNA preparation

Tumor tissues from CRC patients and corresponding normal mucosal samples used in this study were surgically removed at Okayama University Hospital as described previously (3). Tissue samples were treated with proteinase K with the concentration of 1–2 μg/μl at 50–55°C overnight or 24 h before phenol–chloroform procedure. Genomic DNA was extracted using the standard phenol–chloroform procedure, precipitated by ethanol and dissolved in TE buffer.

Bisulfite modification

Bisulfite modification of DNA samples (up to 1 μg) was performed using the CpGenome DNA modification kit (Intergen, Purchase, NY). The modified DNA was used immediately or stored at −20°C for further analysis.

Bisulfite nucleotide sequencing procedure

Nested PCR was performed to amplify a region within the exon 1 of the *MGMT* promoter from nt 685 to 1195 (16). The nucleotide numbering refers to accession nos. HSMETDMET and HSU95038. In the presence of 10–40 ng of bisulfited DNA as a template, first-round PCR was carried out using sense primer 1 [5′-GGTATAGAGTTTTAGG**C**GGAAGTT-3′ (nt 675–698)] and antisense primer 2 [5′-CAACACCTA-CAAAACCACTC**G**AAA-3′ (nt 1177–1200)]. To obtain products for sequencing, a second set of PCR was run with the use of methylation-specific sense primer 3 [5′-TTTAGG**C**GGAA-GTTGGGAGG**C**GT**C**-3′ (from nt 685 to 708)] and antisense primer 4 [5′-CCTACAAAACCACTC**G**AAACTACC-3′ (from 1172 nt to 1195)]. With these primers, the methylated allele in the first PCR product can be amplified. With the use of unmethylated-specific sense primer 5 [5′-TTTAGG**C**GGAA-GTTGGGAGG**T**GT**T**-3′ (from nt 685 to 708)] and antisense primer 4, unmethylated alleles can be amplified. The positions of bisulfite-induced deamination of cytosine residues in the primer sequences are shown in bold.

Amplification consisted of 37 cycles, with denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 40 s. The initial denaturation was at 94°C for 5 min. Finally, the PCR mixture was incubated at 72°C for 10 min. An aliquot of 15 ng of the first round of PCR products was used for a second-round amplification under the conditions described above. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Subsequently, second-round products were cloned into the pGEM-Teasy vector (Promega, Madison, WI) following the manufacturer's protocol. The plasmids were amplified in *Escherichia coli* JM109 (Toyobo, Osaka, Japan) and were purified by a Plasmid purification kit (Qiagen). PCR products from these plasmids, originated from fully-, partially- and non-methylated DNA, were verified by sequencing as the bisulfite-modified nucleotide alteration status. In order to label those originally as fully-, partially- and non-methylated targets, PCRs were performed using biotinylated forward and reverse primers with the same condition as used above, followed by the verification of the sequence with ABI 310 genetic analyzer.

PCR amplification of clinical samples for microarray analysis

PCRs were performed using biotinylated forward and reverse primers with a sample DNA as template. The PCR condition was same as that used above, except for the use of biotinylated forward (5′-TTTAGG**I**GGAAGTTGGGAGG**I**GT**I**-3′) and reverse (5′-CCTACAAAACCACTC**I**AAACTACC-3′) primers. Subsequently, biotinylated PCR products were purified using the Qiaquick PCR purification kit. The products were also examined on the 1% agarose gel.

Microarray procedure

Non-modified oligonucleotides were spotted in duplicate on CarboStation^TM plastic slide (Nisshinbo Industries Inc., Chiba, Japan) with spots of ∼250 μm in diameter and a spot-to-spot distance of 600 μm using a customized microarray robot (13). To analyze each CpG position, two oligonucleotides (spotted at 50 μM solution), reflecting methylated and unmethylated

status of the CpG dinucleotides, were immobilized on the slide according to the manufacturer's procedure. The oligonucleotides were designed to match the bisulfite-modified DNA fragments.

Each biotinylated PCR product was dissolved in distilled water and denatured for 1 min at 95°C. Uni-Hyb hybridization solution (Telechem International Inc., Sunnyvale, CA) was added (80% v/v Uni-Hyb hybridization solution; total volume 12 µl) and the hybridization solution was applied by capillary action between a slide and cover slip. The slide was incubated for 1 h at 55°C in a closed hybridization cassette. Arrays were washed at room temperature in 2× SSC for 5 min. Subsequently, a color development reaction on the slide was performed according to the ABC method utilizing a 4,4,4′,4′-tetramethyl benzidine solution for visualization (14,15). After 15 min, color development reaction was terminated by washing the arrays with distilled water.

Signal detection and data analysis

The arrays were imaged on a GT8700-F scanner (Epson, Nagano, Japan). Signals on arrays were converted to TIFF images, and the signal intensities were quantified by an ImageJ software from the National Institutes of Health. The ImageJ software is freely available on the website (http://rsb.info.nih.gov/nih-image/Default.html). The analysis was performed by selecting a circular region of pixels and determining the values along the perimeter. The mean hybridization intensity of these pixels was then calculated. Unless stated otherwise, the average signal values were taken from two spots on two slides proceeded in parallel.

RESULTS

Detection principle

Figure 1 outlines our strategy for the DNA methylation analysis. In order to distinguish between methylated and unmethylated state of the CpG sites in target sequence, DNA from all samples was first treated with bisulfite, converting unmethylated cytosine to uracil, while conserving methylated cytosine. The region of interest was then amplified by PCR with biotinylated primers, converting originally unmethylated CpG dinucleotide to TpG, while conserving originally methylated CpG. Biotinylated target DNA was then hybridized to the arrayed oligonucleotide probes, specifically designed to distinguish converted from unconverted CpG sites. The ABC method allowed subsequent attachment of avidin–biotinylated peroxidase conjugating onto the DNA chain through specific binding between biotin and avidin (14,15). Subsequently, the addition of 4,4,4′,4′-tetramethyl benzidine generated an insoluble purple signal on the slide surface (14,15).

Microarray-based methylation analysis

We examined the feasibility of our strategy by assessing the methylation status of 12 CpG sites located in the exon 1 of the *MGMT* gene (Figure 2A). A group of eight-arrayed oligonucleotides (19 nt in length) was designed to test the methylation status of 12 CpG sites within the promoter region. Each set contained a pair of methylated and unmethylated
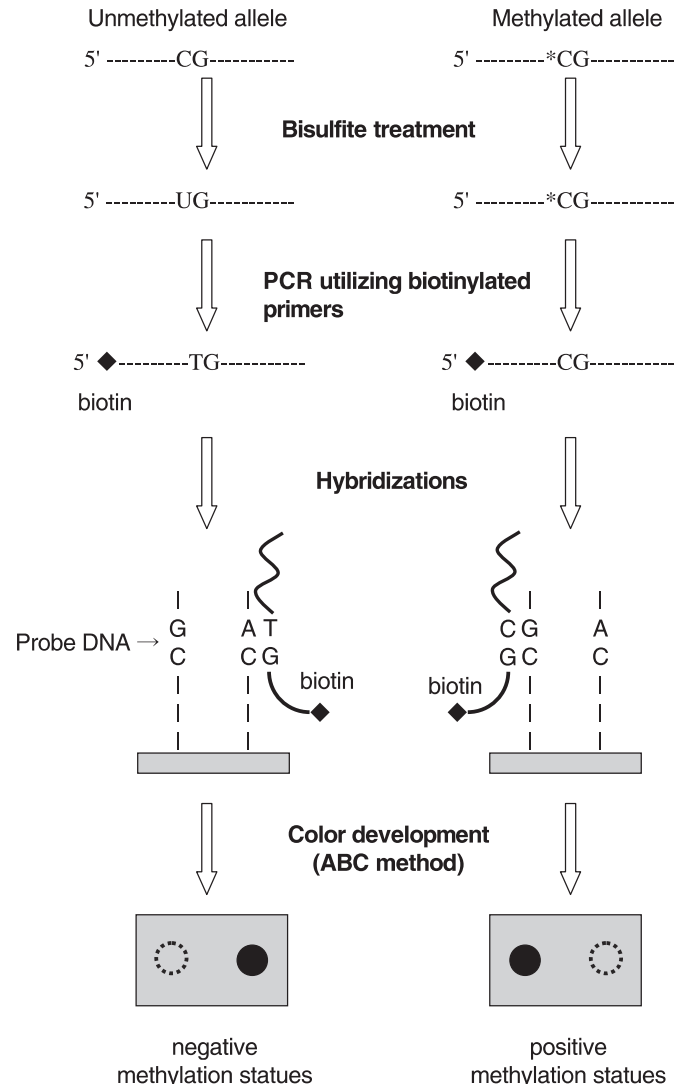

Figure 1. Methylation microarray procedure for the analysis of hybridization data. Genomic DNA was treated with bisulfite and amplified for a specific region of interest containing multiple CpG islands by PCR. PCR product was labeled with biotin and hybridized to the oligonucleotide arrays. Hybridization signals on the array were visualized by the ABC method.

oligonucleotides for interrogating 2–4 CpG sites in close proximity (Figure 2B). First, control DNA from fully-methylated and non-methylated samples was used to test the accuracy and reproducibility of our system.

To exclude experimental variability, the oligonucleotide arrays used for these comparative assays were all derived from the same quality-controlled spotting batch. To quantify the target/probe hybridization sensitivity and linearity, methylation arrays were manufactured with a fixed amount of an oligonucleotide. Then, the arrays were hybridized with mixtures of biotinylated DNA targets from fully-methylated and non-methylated samples at different proportions representing 0, 25, 50, 75 and 100% of DNA methylation. Figure 3A shows a typical image of hybridization signal intensities from these experiments. Irrespective of methylation proportions, hybridization was specific to the

A

cggatatgctgggacagcccgcgcccctagaa**cg**ctttg**cg**tcc**cga**c**g**cc**cg**cag
　　　　　　　　　　　　　　　　　　　　1　　　　2　　　3　　4　　5

gtcct**cgcg**gtg**cg**cac**cg**tttgcgacttggtgagtgtctgggt**cg**cct**cg**ctcc**cg**ga
　　　　6 7　　　8　　　9　　　　　　　　　　　　　　　10　　11　　12

B

Probe No.	*MGMT* CpG locus	Oligonucleotide sequence	T_m (°C)
1	1, 2	M 5' A**CG**CAAAA**CG**TTCTAAAAA 3'	64.3
		U 5' A**CA**CAAAA**CA**TTCTAAAAA 3'	60.0
2	2, 3, 4, 5	M 5' CTA**CG**AA**CG**T**CG**AAA**CG**CA 3'	72.9
		U 5' CTA**CA**AA**CA**T**CA**AAA**CA**CA 3'	64.3
3	6, 7, 8, 9	M 5' CAAA**CG**ATA**CG**CAC**CGCG**A 3'	75.1
		U 5' CAAA**CA**ATA**CA**CAC**CACA**A 3'	66.5
4	10, 11, 12	M 5' CTCTTC **CG**AAAA**CG**AAA**CG** 3'	70.8
		U 5' CTCTTC**CA**AAAA**CA**AAA**CA** 3'	64.3

Figure 2. Promoter sequence of human *MGMT*. The 12 CpG sites located in exon 1 tested in this study were underlined and shown in bold (**A**). The sequence including CpG sites and the T_m of the methylated and unmethylated oligonucleotide probes are shown (**B**).

complementary probe DNA. The average intensity of hybridization signals for the methylated (M) and unmethylated (U) alleles was derived from the duplicate spots. The intensity ratio was calculated by M/(M + U). In all the cases, a linear relationship (R^2 = 0.948–0.988) was observed (Figure 3B). This indicates that DNA methylation and the intensity ratios in the control samples increased proportionally, allowing the determination of the methylation status for the test materials in question.

To further investigate whether our system can be applicable to the mosaic methylation patterns, we prepared a set of PCR products derived from genomic DNA with mosaic methylation patterns, and the PCR products were then hybridized to the arrays. We used the standard curves derived from the aforementioned calibration controls as shown in Figure 3B to determine the methylation level in individual sample DNA, and compared the results with those obtained by bisulfite sequencing (Figure 4A). The percentage of methylation levels with bisulfite sequencing was calculated by dividing the total number of methylated sites at each probe locus by the total number of corresponding CpG loci analyzed and then multiplying by 100. However, as shown in Figure 4B, our system sometimes quantitatively underestimated the methylation levels of partially methylated samples [i.e. the methylation level of probe 2 with target (ii) and the methylation levels of probes 2–4 with target (iii)] compared with the sequencing results. Methylation levels measured by the microarray, however, still showed a relatively good correlation (R^2 = 0.776) with the data obtained from bisulfite sequencing. This discordance may be owing to the fact that the intensity and specificity of the hybridization signal can intricately vary depending on the number and position of the base pair mismatches at each probe, as described previously (17). Accordingly, precise assessment of the

methylation status of partially methylated samples is technically a little more challenging.

Microarray analysis utilizing clinical samples

To apply and assess the general performance of our microarray strategy to DNA methylation analysis, we then analyzed the methylation status of DNA in CRC tissues and the corresponding normal mucosal samples from 12 patients, in which the methylation status of *MGMT* was already known (3). The biotinylated target DNA was prepared from corresponding bisulfite-treated genomic DNA (see Materials and Methods) in order to avoid extremely labor-intensive and time-consuming sample cloning.

Figure 5 shows the methylation levels of 12 CRC tissues and 12 corresponding normal mucosal tissues determined by the microarray. With the use of the standard curves derived from the aforementioned calibration controls (Figure 3B), extensive methylation of the *MGMT* CpG sites was observed in CRC tissues and little or no methylation was detected in normal mucosal samples (Figure 5). These microarray results we examined were generally consistent with a previously determined methylation status by MSP method (3,18).

To further validate our microarray findings in normal and tumor samples, we compared these microarray outcome with that of the bisulfite sequencing of the cloned PCR products from the same samples. Figure 6 shows the methylation status of 12 CpG pairs in the *MGMT* promoter of normal and tumor tissue samples determined by bisulfite sequencing. Although bisulfite sequencing showed extensive methylation in the tumor samples comparative with the microarray results, several samples showed underestimated results by the microarray system compared with those by bisulfite sequencing. This was
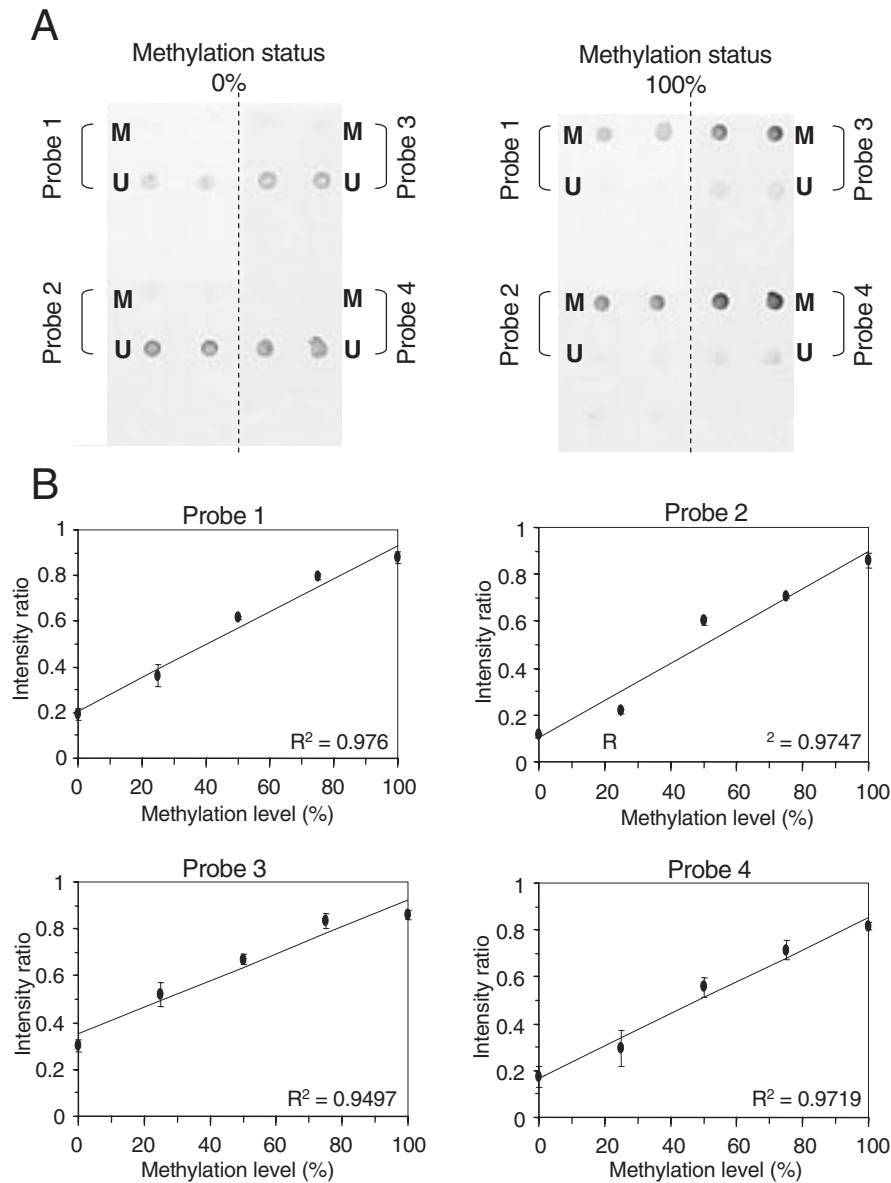
Figure 3. Methylation analysis and quantification of the 12 CpG sites in exon 1 of *MGMT* gene by oligonucleotide microarray. (**A**) Methylation status of *MGMT* identified by oligonucleotide microarray hybridization. Visualized signals by the ABC method are shown. Samples with full-methylation (100%) and non-methylation (0%) were clearly distinguished by the system. (**B**) Quantification of four different level of methylation (0, 25, 50, 75 and 100%) prepared by mixing of full-methylation and the non-methylation samples determined by the signal intensity. The intensity ratios (*y*-axis) represent signal intensities of M/(M + U). The nucleotide position of these CpG sites in each probe is shown in Figure 2B. The error bars indicate SD.

probably due to the unmethylated CpG sites within the target alleles—they might have decreased the methylation levels by disturbing hybridization as shown in Figure 4B. Bisulfite sequencing with methylated-specific primers showed a high level of methylation in sample 286N within the sequence analyzed (Figure 6) and were discordant with the microarray outcome (Figure 5). This may indicate that the microarray analysis might fail to detect an *MGMT* methylation in part of the normal epithelium in 286N sample. Conversely, in the case of 291N, we could not find any methylation within the promoter region by the bisulfite sequencing (Figure 6), whereas the microarray analysis identified weak methylation in part of the *MGMT* promoter (Figure 5). A previous study,

however, indicated the absence of methylation in sample 286N and the presence of methylation in sample 291N by the bisulfite-PCR-single-strand DNA conformation polymorphism analysis (18). This discrepancy can be explained by the different CpG islands targeted by each methodology.

DISCUSSION

In this study, we described a simple and rapid technique to analyze DNA methylation status achieved by the combination of bisulfite DNA treatment, oligonucleotide microarray and ABC detection system. Our alternative microarray system

A

Target No.	Methylation status										
	Probe 2				Probe 3				Probe 4		
	2	3	4	5	6	7	8	9	10	11	12
(i)	M	M	M	M	M	M	M	U	M	M	M
(ii)	M	M	U	U	U	U	M	M	M	M	M
(iii)	M	M	U	U	M	M	U	M	M	M	U

M: Methylated cytosine
U: Unmethylated cytosine

B
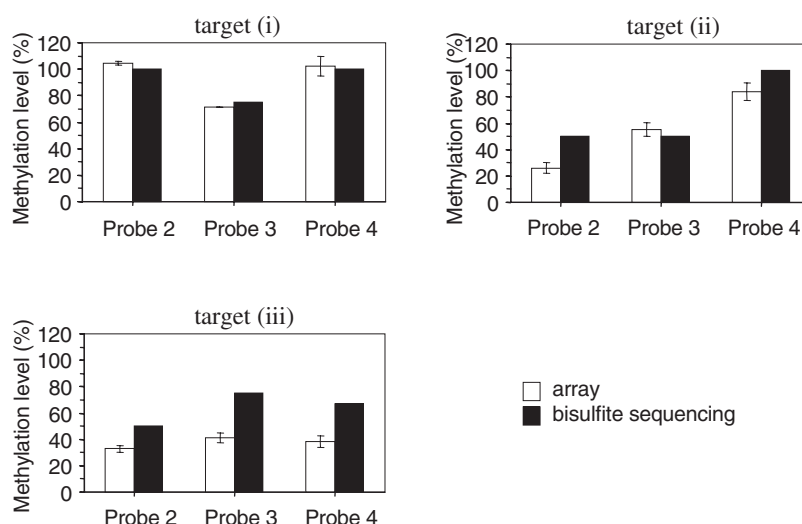
Figure 4. Methylation status of CpG sites in partially methylated samples (i–iii) were determined by bisulfite sequencing (**A**). Probes 2–4 cover the 10 CpG sites in these samples. (**B**) The methylation levels detected by the oligonucleotide microarray were compared with those derived from bisulfite sequencing. The methylation levels by bisulfite sequencing was determined by dividing the total number of methylated CpG sites analyzed by the number of CpG sites at that locus and multiplying by 100, whereas the percentage of methylation levels analyzed by microarray analysis was determined by the use of standard curves derived from the aforementioned calibration controls as shown in Figure 3B. The average signal intensity was taken from two spots on four slides proceeded in parallel. The error bars indicate SD.

enables us to map the methylation status of CpG sites within the *MGMT* promoter of DNA derived from both cultured cells and clinical samples (3).

One possible drawback of our system can be the narrow dynamic range of our image scanning system that may limit the quantitative determination. Although the fluorescent system commonly used in microarray analysis in combination with the expensive readout laser scanner is able to read the images with 65 535 tones (19), the commercially available scanner used in this study was originally developed for importing images from papers or pictures and read the images of only 255 tones. Nonetheless, this study showed that our microarray system could quantitatively detect the methylation status of the fully- or non-methylated DNA (Figure 3B). Sensitivity can be increased by the use of biotin–dCTP instead of dCTP during PCR in our microarray system because all samples are finally prepared by PCR. Also, the biotin–avidin alkaline phosphatase indicator system, which is very convenient and easy to use, was employed to increase the sensitivity. This indicated that our microarray system is reliable enough to discern promoter hypermethylation event that occur in cancer-related genes.

In initial study of these microarray assays, cross-hybridization between imperfect-match probes and targets was sometimes observed (data not shown), which can be observed in other oligonucleotide microarrays. In order to design optimal sequence composition for each oligonucleotide probe, we realized that at least two CpG sites should be included in each probe in our assay. However, as shown in Figure 4B, strict quantification of methylated CpG sites was sometimes hard if the target promoter has mosaic methylation patterns. In addition, this method could not distinguish partially methylated DNA and heterogeneous sample consisting of fully- and non-methylated DNA. Nevertheless, for the purpose of assessing gene silencing in the clinical samples,
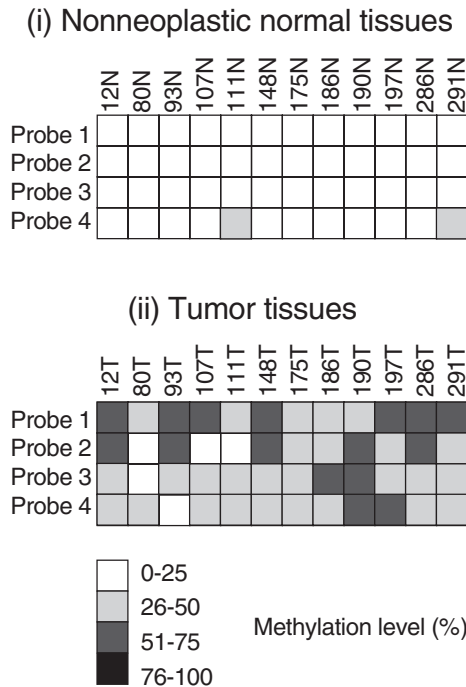
(i) Nonneoplastic normal tissues

(ii) Tumor tissues

	Methylation level (%)
☐ 0–25	
▨ 26–50	
▨ 51–75	
■ 76–100	

Figure 5. Methylation analysis of 12 CpG sites in *MGMT* promoter on DNA extracted from human CRC tissue and counterpart non-neoplastic normal mucosa by oligonucleotide microarray. The nucleotide positions of these sites are shown in Figure 2B.

methylation density in promoter CpG islands is more important than specific methylation that occur at single CpG island (20,21).

In the present study, we compared our methylation microarray system with the bisulfite-sequencing method through the analysis of *MGMT* promoter. Our array method successfully demonstrated methylation status of individual samples comparable with those obtained by the conventional bisulfite sequencing. However, we observed a few samples that revealed different methylation status between the two methodologies. Although the bisulfite sequencing provides complete and reliable methylation profiles of each CpG site, it requires extremely labor-intensive and time-consuming cloning prior to sequencing procedures and this may limit its ability to high-throughput sample analysis. Commonly used MSP has also limited its utility by hybridization specificity of primers and, thus, potentially gives false-positive and false-negative results. Therefore, a simple, robust and inexpensive alternative method, which allows quantitative high-throughput assays to determine the exact location responsible for the silencing of the gene promoter, especially for clinical samples, is desired. Our methylation-array system may fulfill this requirement. Although the precise experimental design is a prerequisite, the results derived from a number of samples in comparison with bisulfite sequencing strongly support the proposition that our precise microarray system can determine the actual methylation levels.

In conclusion, the present technique can be readily reconfigured for high-throughput analysis of DNA methylation. Although our technique needs further improvement in its application to the high-throughput system, we believe that
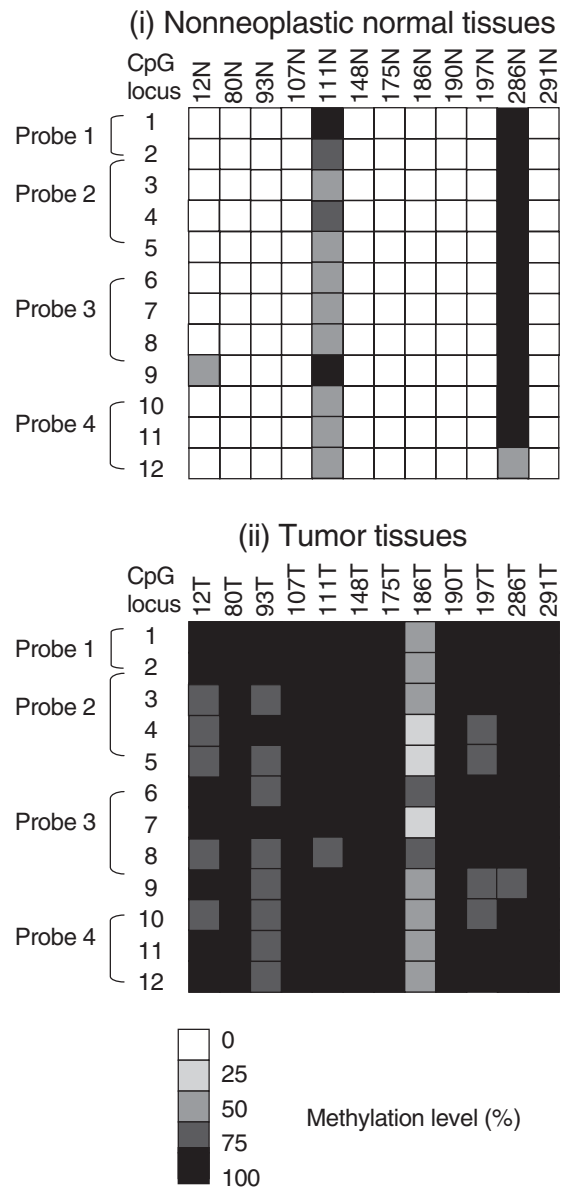
(i) Nonneoplastic normal tissues

(ii) Tumor tissues

	Methylation level (%)
☐ 0	
▨ 25	
▨ 50	
▨ 75	
■ 100	

Figure 6. Methylation analysis of 12 CpG sites on *MGMT* promoter by bisulfite sequencing. Plasmid DNA from positive recombinant clones was isolated and the inserts were sequenced. The nucleotide positions of these CpG sites are shown in Figure 2A and B. The fraction of clones with methylated cytosine in each CpG sites (% methylation) is shown. Each value is derived from four clones.

this technique may contribute to the comprehensive understanding of the cancer epigenetics, as well as the clinical applications.

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